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(54) **Ultrapure hyaluronic acid and method of making it.**

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Description

The present invention is concerned generally with the preparation, purification and use of hyaluronic acid and its salts and specifically with the preparation of hyaluronic acid from a microbiological source.

Hyaluronic acid is a naturally occurring high molecular weight polysaccharide having an empirical formula of $(C_{14}H_{20}NNaO_{11})n$ where n>1000. The general structure of hyaluronic acid is illustrated in Merck Index, Ninth Ed. (3rd printing, 1978), at page 624. It is well known that hyaluronic acid and its salts, hereafter collectively referred to as HA, can be obtained from at least three sources: human umbilical cords, rooster combs and certain bacterial cultures such as group A and C hemolytic streptococci. To the best of our knowledge, however, only umbilical cords and rooster combs are used as sources for commercially available HA. This is somewhat surprising in view of certain disadvantages associated with using those two sources (e.g. relatively low yields, contamination with chondroitin sulfate, and labor intensive processing and purification steps).

Since HA is found in aqueous and vitreous humor and the synovial fluid of mammalian joints, there has been considerable interest in obtaining purified HA for use as a fluid replacement to correct pathological conditions in the eye and in joints. The preparation of HA from rooster combs and human umbilical cords and its use in eye and joint applications is described in U.S. Patent 4,141,973 to E. A. Balazs. That patent also provides a detailed review of the technical literature describing the isolation, characterization and uses of HA.

U.S. Patent 4,303,676, also to E. A. Balazs, describes cosmetic formulations containing sodium hyaluoronate fractions in various molecular weight ranges made from rooster combs. U.S. Patent 4,328,803 to L. G. Pape discloses the use of an ultrapure hyaluronic acid salt in eye surgery. The HA product used was a sodium hyaluronate salt available under the registered trademark Hyartil® from Pharmacia, Inc. and obtained in commercial quantities from rooster combs.

The only literature found which describes extraction of hyaluronic acid from bacteria (see Kjem and Lebech, Acta Path, Microbiol. Scand. Sect. B, *84*:162—164, 1976) uses a media and process which are unacceptable for some purposes. The described media will not support growth of most Streptococci. The described process begins with heat killing the Streptococci. This extracts the organisms, releasing numerous internal contaminants which are more likely to be reactive and which are difficult to remove from the final product. Therefore, it is likely the resulting HA could not be used for injcetion into mammals.

Because the medical applications of HA require that the HA be injected into a mammalian body (e.g. as a fluid replacement), it is very important that the injected products be as pure as possible to avoid reactivity problems. This importance of purity is described in U.S. Patent 4,141,973 which describes an ultrapure HA product prepared from rooster combs or, alternatively, from human umbilical cords. In addition to purity, it appears that control of molecular weight of an HA product is very important (e.g. the 4,141,973 patent suggests an average molecular weight of at least 750,000 and U.S. Patent 4,303,676 suggests having two distinct fractions of controlled molecular weight, one low and one high). U.S.—P. 2,975,104 describes a hyaluronic acid obtained from bacterial source but having a high viscosity.

Chem. Abstracts. Vol. 82 (1975) 121419 v describes hyaluronic production from bacterial source without giving purity characteristics of the thus obtained product. Although there is a description of a high molecular weight (1,200,000) HA preparation of a very high purity (i.e. less than 0.05% protein) in a paper by Swann, Arch. Opthal. *88*, pp. 544-8 (1972), we are unaware of any description of an HA product having the following advantages: (1) derivable from a microbiological source at relatively low costs, in high yields, and with low reactivity upon injection; (2) having a desirably high and closely controlled average molecular weight; and (3) being substantially free of protein and nucleic acid impurities. Quite surprisingly, we have found it is now possible to prepare such a product. Details of its preparation, characterization, and use are described below.

The method of preparing an ultrapure HA preparation of controlled high average molecular weight comprises the steps of culturing an HA-producing, hyaluronidase-negative or hyaluronidase-inhibited organism under conditions sufficient to enhance HA content in the culture, releasing the HA from the cells, and purifying the released HA to remove substantially all protein and nucleic acids.

In preferred embodiments, the HA is prepared from a group C streptococcal organism (such as *Strep. equi*), preferably cultured in a medium free of extraneous proteins, with the final product being a sterile preparation having a tightly controlled average molecular weight and containing less than about 1.25 mg/ml of protein (preferably less than about 0.10 mg/ml) and less than about 45 µg/ml of nucleic acids (preferably less than about 5 µg/ml), and is used (for example) as a synovial fluid replacement in mammals.

As shown in the examples below, the HA product of this disclosure is different from commercially available HA products in that it is made from a hyaluronidase-negative or hyaluronidase-inhibited microbiological source, has a tightly controlled average molecular weight and, very importantly, is substantially free of proteins and nucleic acids, and contains no chondroitin sulfate, all of which are considered undesirable contaminants in any product intended for replacement of an animal fluid. As used herein, the expression "substantially free of", when applied to the protein and nucleic acid content of an HA preparation, means that the pr tein content of the product is less than about 1.25 mg/ml (preferably less than about 0.10 mg/ml) and the nucleic acid content is less than about 45 µg/ml (preferably less than about 5 µg/ml). The expression closely controlled high molecular weight means that at least 98% of the HA is

within a given high av rage molecular weight range (preferably from about 2.0 million (MM) to about 4.0 MM, and represented by an essentially single, substantially symetrical molecular weight distribution peak via the HPLC technique described below). Hyaluronidase-negative means that no measurable amounts of extracellular hyaluronidase (able to degrade HA to small molecules) are associated with the organism. Hyaluronidase-inhibited means that an inhibitor such as heat or enzyme inhibitors has been used so as to eliminate the breakdown of HA to smaller molecules.

In the examples below, the purity and efficacy of the HA prepared according to this disclosure was demonstrated and compared with existing commercial HA products by using the HA as a joint fluid replacement in the horse. It can be appreciated that the product can also be used in any applications in other mammals, including humans, which call for the use of a highly purified HA preparation as a fluid replacement or for other purposes such as cosmetics.

In the specific illustrative preparation steps given below, we used a known group C streptococcal HA producer (*Strep. equi*) and, by novel culturing and purification techniques, we demonstrate how it is now possible to obtain an ultrapure product of controlled molecular weight with very large yields (e.g. Avg. 31% w/w vs 0.079% w/w from rooster combs), thus providing the advantages of higher purity and better product molecular weight control with the clear advantages of economy. A sample of the *Strep. equi* strain used below has been deposited with the American Type Culture Collection, Rockville, MD 20852 as A.T.C.C. No. 39,506.

As noted above, it has long been known that HA is a major component of the capsule of Types A and C streptococci. A representation of a streptococcal organism illustrating the capsular location (hyaluronate capsule) of HA has been published by Beachey and Stollerman and is shown in *Trans Assoc. Am. Physicians Phila., 85*:212—221, 1972.

As has been demonstrated, the HA capsule is the outermost component making up a large portion of the total streptococcal cell. During growth of a streptococcal culture such capsule may be observed after india ink staining of the culture as a bright halo around each cell. As a result of this procedure it has been determined that maximum capsule production is obtained from several strains of streptococci grown in controlled fermentation systems by about 4 to 6 hours after the beginning of log phase growth. During late log and stationary phase the visible capsule disappears. It is known that in some strains of streptococci this disappearance of capsule is due to enzyme degradation by hyaluronidase. In long-term fermentation studies (2—5 days growth) with at least one group C streptococci (*Strep. equi*) in which pH, temperature, and glucose levels were controlled, we have determined that yields of HA can be substantially increased even though, surprisingly, the capsule is not visibly apparent in the culture. In such studies it was concluded that this strain probably lacks hyaluronidase. Thus, when extracellular hyaluronidase negative strains of streptococci are grown under controlled conditions specific for HA production, yields of an extraordinarily pure, high specific molecular weight HA have been reached and this lack of th hyaluronidase enzyme is considered an important aspect of the HA preparation.

As indicated, this invention describes the process for obtaining such high yields of extraordinary quality, high molecular weight hyaluronic acid from bacteria such as Streptococci and a method of use of such HA to replace synovial fluid from diseased joints in order to reduce lameness and swelling of such joints.

In our best examples, fermentation of a Group C streptococcus was continued at a pH between 7.0 and 7.2 for from 24 hours to 120 hours at 37°C. A special chemically defined media, described by I. van de Rijn in *Infect. and Immun., 27*:444—448, 1980, was used for growth. This media is preferable since it contains no extraneous proteins which would have to be removed in later purification steps. Dextrose is added at 24 hour intervals to serve as a carbon source. The culture may be grown under intermittent pH control, adjusting to pH 7.6 at each addition of dextrose. Approximately 12 hours before harvest, the pH controller is shut off and the pH is allowed to drop to 6.5 to 6.8 where the culture stops growing. This allows more efficient centrifugation and somewhat better yields of hyaluronic acid.

At harvest, at least 0.01% sodium lauryl sulfate (SLS), or an equivalent anionic detergent, is added t the culture in order to release hyaluronic acid from the cells. After at least 15 mins., the SLS culture is titrated for floc formation after addition of varying amounts of a 10% solution of hexadecyltrimethylammonium bromide, or equivalent cationic detergent.

Generally, between 100 ml and 400 ml of this second detergent is added to 10 l of SLS culture in order to precipitate HA and SLS. After allowing at least 1 hour for maximum floc formation the precipitate is collected via centrifugation or sieve filtration. This precipitate is then solublized in 2M $CaCl_2$ of approximately 1/10 to 1/20 the original volume. Solubilization is carried out for at least 6 hours at 4°—30°C. The resulting suspension is centrifuged or sieve filtered in order to remove the precipitate which contains cellular contaminants and both detergents. The supernate is saved and extracted with 2 volumes of a suitable alcohol (95% EtOH or 99% isopropanol preferred). A gelatinous precipitate forms which is collected after at least 1 hour via centrifugation or sieve filtration. The precipitate is solubilized overnight at 4°C—10°C in deionized, distilled water approximately 1/10 to 1/20 the original volume. The suspension is centrifuged or sieve filtered to remove the precipitate. One percent NaCl (w/v) is added to the supernate and dissolved. Then, 2 volumes of an appropriate alcohol are added to reprecipitate the HA. Such precipitate is allowed t settle at least one hour after which it is collected via centrifugation or sieve filtration.

The solubilization of the HA in water followed by 1.0% NaCl addition and alcohol precipitation are continued in increasingly smaller volume (1/20—1/100 original volume) until the HA-water solution is clear. This usually requires at least four additional alcohol precipitation steps. An outline of the process is sh wn below.

Outline of process for extraction of bacterial hyaluronic acid

1. Grow streptococcus organism
2. 1 ml/l SLS 10%
3. 10—40 ml/l
   Hexadecyltrimethylammonium bromide 10%
4. Collect ppt.
5. Solublize in 2M $CaCl_2$
6. Collect supernate
7. 2 Vol. alcohol
   (ppt. HA, some nucleic acids, some protein)
8. Collect ppt.
9. Solubilize ppt. in DI—H_2O
10. Discard undissolved ppt.
11. Collect supernate
12. 1% NaCl